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Use of electron microscopy in the examination of lattice defects in crystals of alcohol oxidase

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Alcohol oxidase, purified from the yeast *Hansenula polymorpha*, was crystallized in vitro for the purpose of determining its structure at atomic resolution by X-ray diffraction methods. The crystals obtained yielded only extremely weak diffraction patterns: the maximal resolution observed was in the best case 6 Å. Electron microscopy of thin sections indicated that most crystals showed lattice defects which might explain the poor diffraction patterns: most surprising was the appearance of large holes interrupting an otherwise regular lattice in one of the crystal forms examined. Our results indicate that transmission electron microscopy is a suitable tool for the inspection of crystals to be used in X-ray crystallography.

The method allows rapid determination of lattice defects and enables optimization of crystallization conditions.

Alcohol oxidase; Protein crystallization; Ultrastructure; (*Hansenula polymorpha*)

1. INTRODUCTION

The first task of any structure determination by X-ray crystallographic methods is finding appropriate conditions for producing large single crystals which diffract to high resolution. Occasionally, the crystallographer encounters promising crystals which appear regularly shaped when examined by light microscopy, but nevertheless diffract either very poorly or not at all. It is then important to consider the nature of the disorder that limits the resolution of diffraction. Such an analysis may indicate a way to improve the crystal order by modification of the original crystallization scheme.

In this report we describe the use of electron microscopy for examination of poorly diffracting crystals of alcohol oxidase from the methanol-

utilizing yeast *Hansenula polymorpha*. Alcohol oxidase is a key enzyme in methanol metabolism in yeasts and converts methanol into formaldehyde and hydrogen peroxide [1]. The active form of the enzyme is an oligomer of approx. 600 kDa consisting of eight identical subunits, each of which contains an FAD prosthetic group [2]. In intact cells this enzyme is localized in subcellular organelles, called microbodies [1]. Under certain growth conditions alcohol oxidase octamers are organized into crystalloids which have a regular, open lattice structure [3].

In several in vitro conditions, purified alcohol oxidase also readily forms crystalline structures, however, we have not been able to obtain well-ordered crystals suitable for a structure determination at atomic resolution. We wish to show here that electron microscopy is a suitable tool for the inspection of such crystals and has allowed us to discriminate between conditions which improve crystal order, and conditions which produce major lattice defects.

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2. MATERIALS AND METHODS

2.1. Purification of alcohol oxidase

Purification was performed as described in [4].

2.2. Crystallization of alcohol oxidase

Crystals were grown by vapor diffusion at room temperature using the hanging drop method [5]. Although a full range of precipitating agents was tested, including salts and organic solvents, polyethylene glycol 4000 in the range 2.5 to 5.0% (w/v) was found to produce the most promising crystals in terms of size and shape for X-ray diffraction experiments. Specific growth conditions for the crystals described in this study are given in table 1. When possible, selected crystals were mounted in a standard manner on a Philips sealed tube X-ray unit and still photographs were taken to determine the maximum resolution of diffraction.

2.3. Fixation and postfixation methods

Selected crystals were prefixed in 3% (v/v) glutaraldehyde in 50 mM potassium phosphate buffer, pH 7.0, for 1 h at 0°C. They were subsequently postfixated in 1% (w/v) osmium tetroxide in the same buffer for 1 h and then washed with distilled water. After embedding in 1.5% (w/v) agar, the samples were stained in a 0.5% (w/v) uranyl acetate in distilled water for 2 h, then dehydrated in a graded ethanol series and embedded in Epon 812. Ultrathin sections were cut with a diamond knife, poststained with lead acetate [6] and examined in a Philips EM300.

3. RESULTS AND DISCUSSION

Crystals of alcohol oxidase grown in polyethylene glycol 4000 with various additives (table 1, growth conditions A–D) were examined after embedding, ultrathin sectioning and staining as described in section 2. Representative micrographs corresponding to each growth condition are presented in fig.1(A–D). In each case, the crystals selected for examination by electron microscopy are derived from the same vapor diffusion droplet, and are of the same dimensions, as the crystals selected for X-ray diffraction experiments: in one case, the very same crystal exposed to the X-ray beam was examined by electron microscopy.

In the presence of DTT, a precipitate that appeared to be microcrystalline formed in the crystallization droplets. However, in thin sections of this material no lattice order was detectable (fig.1A). Addition of calcium chloride, on the other hand, resulted in growth of small cube shaped crystals. These were shown to have well ordered regions by electron microscopy, but, surprisingly, with large 'holes' interrupting the lattice (fig.1B). A considerable improvement was obtain-

Table 1
Growth conditions of alcohol oxidase crystals

Growth conditions	Crystal morphology	Maximum resolution of diffraction (Å)
A. 4% (w/v) PEG 4000, 100 mM Bis-Tris-HCl, 5 mM dithiothreitol, pH 6.6	microcrystalline precipitate	–
B. 2.5% (w/v) PEG 4000, 100 mM Tris-maleate, 5 mM CaCl ₂ , pH 7.0	small cubes	20
C. 5% (w/v) PEG 4000, 100 mM potassium phosphate, 10 mM EDTA, pH 6.8	4-pointed stars	20
D. 5% (w/v) PEG 4000, 50 mM potassium phosphate, 2 mM EDTA, pH 8.0	small cubes	6

ed when crystals were grown in the presence of EDTA: these crystals also appeared to have well ordered regions which were interrupted by much smaller holes (fig.1C). However, crystals obtained in the presence of either calcium chloride or EDTA (growth conditions B and C) were not observed to diffract beyond 20 Å resolution in the X-ray beam. Interestingly, ultrathin sections prepared from an EDTA crystal (growth condition C) exposed to the X-ray beam (fig.1F) showed a considerably increased disturbance of the lattice structure, especially in the central part of the crystalloid, suggesting that radiation damage had occurred. Crystals of *Hansenula polymorpha* alcohol oxidase may therefore be insufficiently stable in the X-ray beam to obtain high resolution diffraction patterns.

As a control, a crystal of *Panulirus interruptus* hemocyanin, a protein similar to alcohol oxidase in terms of its molecular size and oligomeric properties (six 75 kDa subunits arranged in a 450 kDa hexamer), was examined by this technique. The hemocyanin crystal was chosen as it is known to have good internal order: its X-ray structure has been determined to 3.2 Å resolution [7]. Micrographs taken from thin sections of such crystals show a regularly ordered lattice with no visible defects, a representative view is shown in

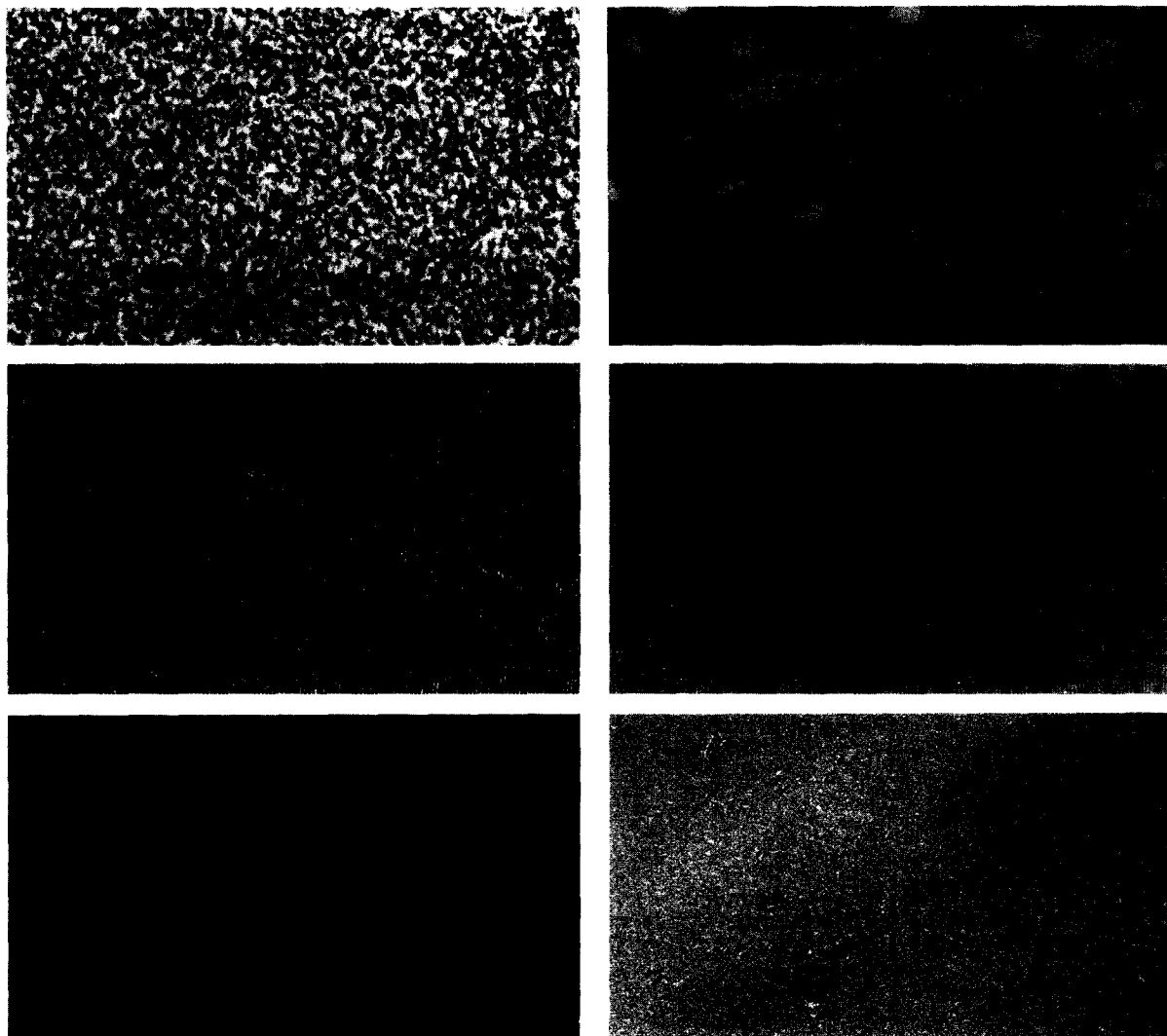


Fig.1. Electron micrographs of ultrathin sections of crystals of alcohol oxidase. (A–D) Micrographs of crystals formed under the conditions mentioned as A–D in table 1, and are shown in the same order. (F) A micrograph of a crystal as in fig.1C, but after exposure to the X-ray beam. (E) Electron micrograph of a *Panilurus interruptus* hemocyanin crystal. All micrographs are printed at the same magnification, namely $42\,300\times$.

fig.1E. We take this as evidence that the electron microscopic preparation procedures do not damage the overall crystal morphology, and that the observed lattice defects in the alcohol oxidase crystals were not artifacts introduced by the methods used.

Fig.1D shows a thin section of a crystal of alcohol oxidase, also grown in the presence of EDTA, but at slightly higher pH. As is evident from this micrograph this crystal shows a virtually

perfect regular lattice of quality similar to that of the hemocyanin crystal. Crystals from the same crystallization droplet were observed to diffract to 6 \AA resolution.

In view of the above results, we feel that electron microscopy is a suitable tool to judge certain lattice defects in crystals which are to be used, or have been used, in X-ray crystallography, and thus enables the investigator to optimize crystallization conditions.

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